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(54) Title: CHROMATOGRAPHIC PROCESS FOR PURIFYING PLASMID DNA USING SUPERPOROUS SUPPORTS

(57) Abstract: The present invention relates to a process for purifying plasmid DNA. Specifically, the present invention is applied in the purification of plasmid DNA with chromatographic techniques, which use insoluble supports. Its main novelty lies in the fact that the supports used are considered superporous supports. The outcome of the chromatographic operations might be largely improved when such supports, say superporous, are used, leading to a significant increase of both the overall process productivity and yield.

DESCRIPTION

"CHROMATOGRAPHIC PROCESS FOR PURIFYING PLASMID DNA USING SUPERPOROUS SUPPORTS"

FIELD OF THE INVENTION

The present invention is related to plasmid DNA purification processes and is aimed in particular to improve the performances of the chromatographic processes used leading thus to significant increases of the productivity and yield of the whole purification process.

BACKGROUND TO THE INVENTION

The interest in purifying large amounts of plasmid DNA has largely increased in the last years owing to the rapid development of gene therapy and DNA vaccination. It is expected that, as a result of these two molecular medicine techniques, it will be possible to cure and/or treat gene-related diseases, such as cancer, cystic fibrosis, Parkinson's disease, or to prevent infectious diseases, such as AIDS and malaria, by immunising the population against infectious agents for which the vaccines are currently non-existent or inefficient.

At laboratory scale, typical plasmid DNA purification processes involve cellular lysis in the presence of enzymes, such as RNase and lysozyme, extractions with organic solvents and ultracentrifugations in density gradients. As well as time consuming, these processes raise several technical and regulatory concerns when used to produce plasmid DNA for therapeutic purposes: on the

one hand, these operations are un-scalable or difficult to scale-up; on the other hand, the use of enzymes to produce therapeutic products is not approved by the regulating authorities. Furthermore, these processes use toxic and carcinogenic reagents such as phenol, chloroform, cesium chloride and ethidium bromide, which is unthinkable when the final product, plasmid DNA, aims therapeutic applications.

Large-scale plasmid DNA purification involves chromatographic operations after cell growth and lysis. The most use chromatographic operations are anion-exchange chromatography and size-exclusion chromatograph, however other types of chromatography, such as reverse phase, hydrophobic interaction, affinity or pseudo-affinity chromatography, can be used in stead of or as well as in a plasmid DNA large-scale purification process.

In short, the process for the production/purification of plasmid DNA at large-scale can be described in the following way: the cellular extract, obtained after harvesting and lysing the cells, is fed to an ion-exchange column at high ionic strength, for example NaCl 0.5 M. Unretained impurities, such as proteins and nucleotides, are removed while genomic DNA and high molecular weight RNA are retained together with plasmid DNA. RNase can be added any time prior to ion-exchange; the cellular lysis might be performed at high temperatures using physical, chemical or enzymatic methods; the extracts fed to the columns might be clarified and concentrated using methods aimed at enriching the plasmid DNA content. These methods include precipitations with chaotropic salts - whose cations may be metallic, alkaline earth or quaternary ammonium elements - and/or polymers such as polyethylene glycol (PEG), dextrane, derivatives or analogues and/or alcohols such as ethanol or isopropanol. Said reagents can be used isolated in different purification steps or together in a single step. The patents WO 99/16869, WO 96/36706 and WO 95/21178 are examples of these treatments.

Retained plasmid DNA is eluted by increasing the buffer ionic strength in gradient or in step, or by substitution. The fractions containing plasmid DNA are pooled after performing a control check by anion-exchange HPLC and/or agarose gels electrophoresis, and can be further concentrated using methods similar to the ones described above. Plasmid DNA is then purified to a level of purity close to 100% by size exclusion chromatography.

The main disadvantage of the chromatographic purification of plasmid DNA is that the supports used have low binding capacities for such molecules. These low capacities force the use of high relative amounts of support, meaning that large columns and poor support utilisation must be used. This is due to the fact that plasmids are large molecules and are excluded from the particle pores. Thus, the adsorption of the plasmids to the supports only occurs at the particle surface and most of the support capacity is unused.

DESCRIPTION OF THE INVENTION

One way of solving the disadvantages mentioned above is to use supports whose particles have pore diameters large enough to accommodate plasmid molecules.

In accordance with the invention, superporous particles can be applied in plasmid DNA purification processes - superporous particles are considered to be particles whose pores are considered macropores according to the IUPAC definition: "Macropores are pores with dimensions greater than 0.5 μm" [Seader, J.D and Henley, E.J. in Separation process principals, chapter 15, page 778-878]. Thus, in the chromatographic steps described above the plasmid is placed in contact with an insoluble matrix composed by superporous particles.

This operation can be carried out in batch or in fixed, expanded or fluidised bed. In this case, the plasmid production/purification process consists of the following steps:

1. Growth and lysing of the cells in order to release the plasmid DNA, thereby obtaining an extract.
2. Clarification of the extract obtained in (1) in order to obtain a clarified extract.
3. Removal of the precipitates obtained in (2).
4. Concentration of the extract obtained in (3) or (1) in order to obtain a concentrated preparation.
5. Removal of the supernatanat obtained in (4) and resuspension of the precipitate in an appropriate buffer - alkaline pH and high ionic strength, i.e. pH=8, 0.5 M NaCl.
6. Contact of the extract obtained in (1) or (3) or (5) with superporous particles derivatised with ligands which retain the plasmid.

The cellular culture may be of prokaryote or eukaryote origin and the plasmid, containing genes encoding for proteins or peptides of interest, may be have various sizes and it may have high or low copy numbe.

Any method of cellular lysis can be used, either mechanical, chemical or enzymatic, provided that the plasmids are not damaged during the process.

Depending on the chromatographic technique used, the superporpous particles can be derivatised with ion-exchange ligands (strong or weak), affinity ligands or pseudo-affinity ligands, reverse phase ligands or hydrophobic interaction ligands, or mixed ligands which retain the plasmid. The superporous particles may not yet be derivatised if they are to be used in size exclusion chromatography.

In a preferred embodiment the particles are derivatised with appropriate ligands, in which the ligands are bound to the particles by means of spacer arms.

The particles used can be manufactured using agarose, cellulose, dextrane, polyacrylamide, polystyrene, polystyrene/divinylbenzene, silica, vinyl polymer, hydroximethyl-methacrylate polymers or combinations or these polymers.

Regarding the pore diameters, it must be pointed out that it is commonly known that plasmid DNA is completely excluded from pores with less than 1 μm average diameter and partially excluded form pores with average diameter in the range 5 to 10 μm. Experimental results demonstrate that plasmid DNA penetrates pores whose average diameter is greater than 30 μm.

DESCRIPTION OF THE FIGURES

Figure 1 represents the adsorption of plasmid DNA onto Q Sepharose High Performance®.

Figure 2 represents the adsorption of plasmid DNA onto Q Sepharose Fast Flow®.

Figure 3 represents the adsorption of plasmid DNA onto Q Sepharose Big Beads®.

Figure 4 represents the adsorption of plasmid DNA onto Streamline QXL®.

Figure 5 represents the adsorption of plasmid DNA onto a superporous support, Cytopore® which have 30 μm average pore diameter, in accordance with the present invention.

EXPERIMENTAL PART

Adsorption isotherms of a 4.8 kb model plasmid onto five different supports were performed in the presence of NaCl 0.5 M at 25°C. Four of these supports are normally used to purify proteins and have been used in plasmid DNA purification processes as well: Q-Sepharose High Performance®; Q-Sepharose Fast Flow®; Q-Sepharose Big Beads®; Streamline QXL®. The fifth support is a superporous support normally used to immobilise cells (Cytopore®). Langmuir isotherms were adjusted to each set of experimental points (Figure 1 to 5) and the respective maximum capacities (Q_{max}) were calculated.

Examples

Assays were performed to study the adsorption of a 4.8 kb plasmid onto the supports tested in accordance with the procedure described in Examples 1 to 5.

Example 1 (comparative)

Adsorption of the plasmid onto Q Sepharose High Performance® (HP)

A plasmid solution is prepared in an alkaline buffer (10 mM TRIS, 1mM EDTA, pH 8) containing 0.5 M NaCl and the absorbance at 260 nm is measured. This measurement enable the quantification of the plasmid

concentration (1 absorbance unit at 260 nm corresponds to a plasmid concentration of 50 $\mu g.ml^{-1}$). The solution obtained is placed in contact with the support and the mixture is incubated for more than 24 hours at 25°C and 250 rpm. The support is removed, dried for 24 hours in an oven and weighed - dry weight. The solution absorbance at 260 nm is measured to determine the amount of plasmid remaining in solution. The amount of adsorbed plasmid is calculated by mass balancing the solution content prior and after the adsorption experiment. The isotherm is the graphical representation of the amount of plasmid adsorbed per unit of support dry weight (q) as a function of the equilibrium plasmid concentration (C). A Langmuir type model is fitted to the experimental data and the adsorption parameters (Qmax and K_l) are determined (Figure 1 and Table 1).

Example 2 (comparative)

Adsorption of the plasmid onto Q Sepharose Fast Flow® (FF)

The procedure described in Example 1 (comparative) is followed using Q Sepharose Fast Flow® instead of Q Sepharose High Performance®. The results are represented in Figure 2 and Table 1.

Example 3 (comparative)

Adsorption of the plasmid onto Q Sepharose Big Beads® (BB)

The procedure described in Example 1 (comparative) is followed using Q Sepharose Big Beads® instead of Q Sepharose Fast Flow®. The results are represented in Figure 3 and Table 1.

Example 4 (comparative)

Adsorption of the plasmid onto Streamline QXL® (SL)

The procedure described in Example 1 (comparative) is followed using Streamline QXL® instead of Q Sepharose High Performance®. The results are represented in Figure 4 and Table 1.

Example 5 (in accordance with the invention)

Adsorption of the plasmid onto a superporous support- Cytopore® (Cyt)

The procedure described in Example 1 (comparative) is followed using a superporous support, Cytopore®, instead of Q Sepharose High Performance®. The results are represented in Figure 5 and Table 1.

RESULTS

Table 1 - Langmuir parameters (Qmax and Kl) calculated for the adsorption equilibrium of a 4.8 kb plasmid onto the supports tested at 0.5 M NaCl and 25°C.

Support	Q_{max} ($mg.g^{-1}$)	K_1 ($l.mg^{-1}$)
HP	20	0.35
FF	6.3	0.33
BB	3.1	0.3
SL	6.2	0.35
Cyt	1240	0.6

The results presented demonstrate that the plasmid adsorption

capacity of the superporous support is much higher than the capacity of the chromatographic supports generally used (Figures 1 to 5). This increase - which is at least 70 times and around 550 times when the capacity of the superporous support is compared to the Q Sepharose High Performance® and Big Beads® support respectively - is due to the large pore diameter of the superporous support, which is large enough to allow the plasmids to enter.

CLAIMS

1. Process to purify plasmids which involves the separation of plasmid DNA using chromatographic techniques which use insoluble supports, characterised by the use of a support containing superporous particles, say with greater than 0.5 μm average particle pore diameter.

2. Process according to claim 1, characterised in that the plasmid DNA is retained in the support and is subsequently eluted.

3. Process according to claims 1 and 2, characterised in that the pores of the particles are macropores with dimensions greater than 1 μm.

4. Process according to claim 3, characterised in that the pores of the particles are macropores with dimensions greater than 5 μm.

5. Process according to claim 4, characterised in that the pores of the particles are macropores with dimensions greater than 20 μm.

6. Process according to any of claims 1 to 5, characterised in that the superporous particles can be derivatised with ion-excahnge ligands (strong or weak), affinity or pseudo-affinity ligands, reverse phase or hydrophobic interaction ligands or mixed ligands, which retain the plasmid, or may not yet be derivatised if they are to be used in size exclusion chromatography.

7. Process according to claim 6, characterised in that the particles are derivatised with appropriate ligands, in which the ligands are bound to the particles by means of spacer arms.

8. Process according to claim 6, characterised in that the particles have anion exchangers.

9. Process according to any of claims 1 to 8, characterised in that the operating mode is continuous, in fixed, expanded or fluidised bed, or in batch.

FIGURE 1

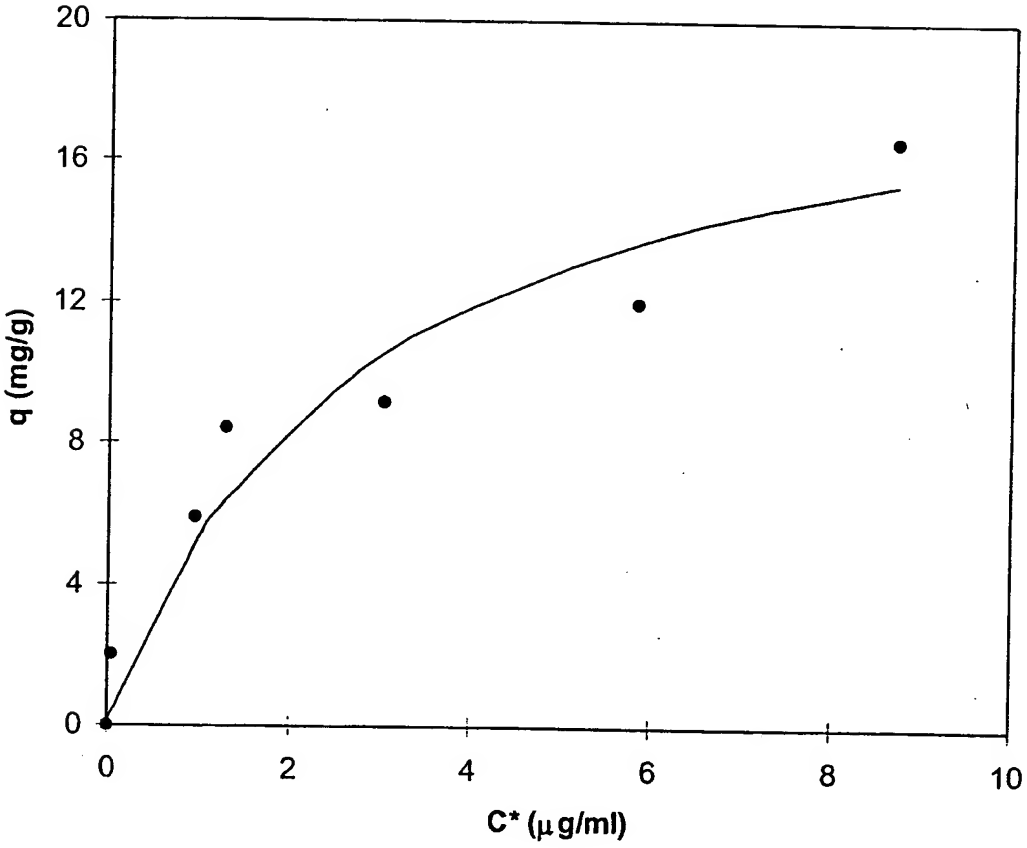


FIGURE 2

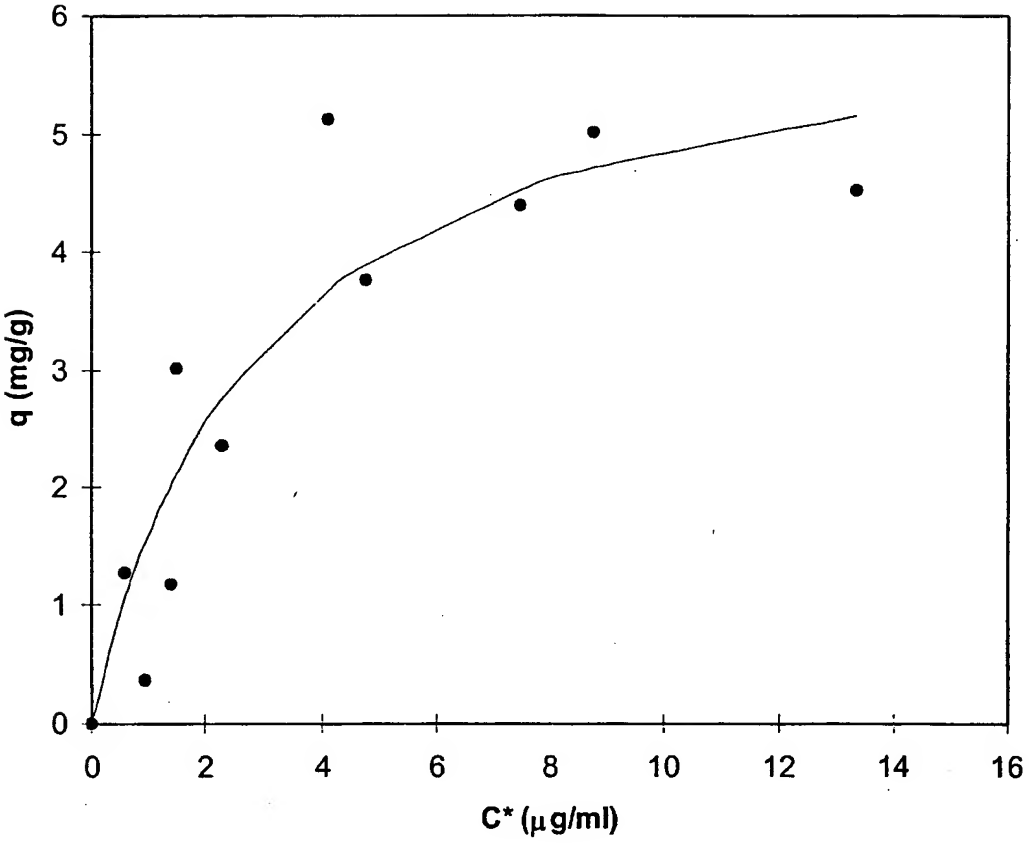


FIGURE 3

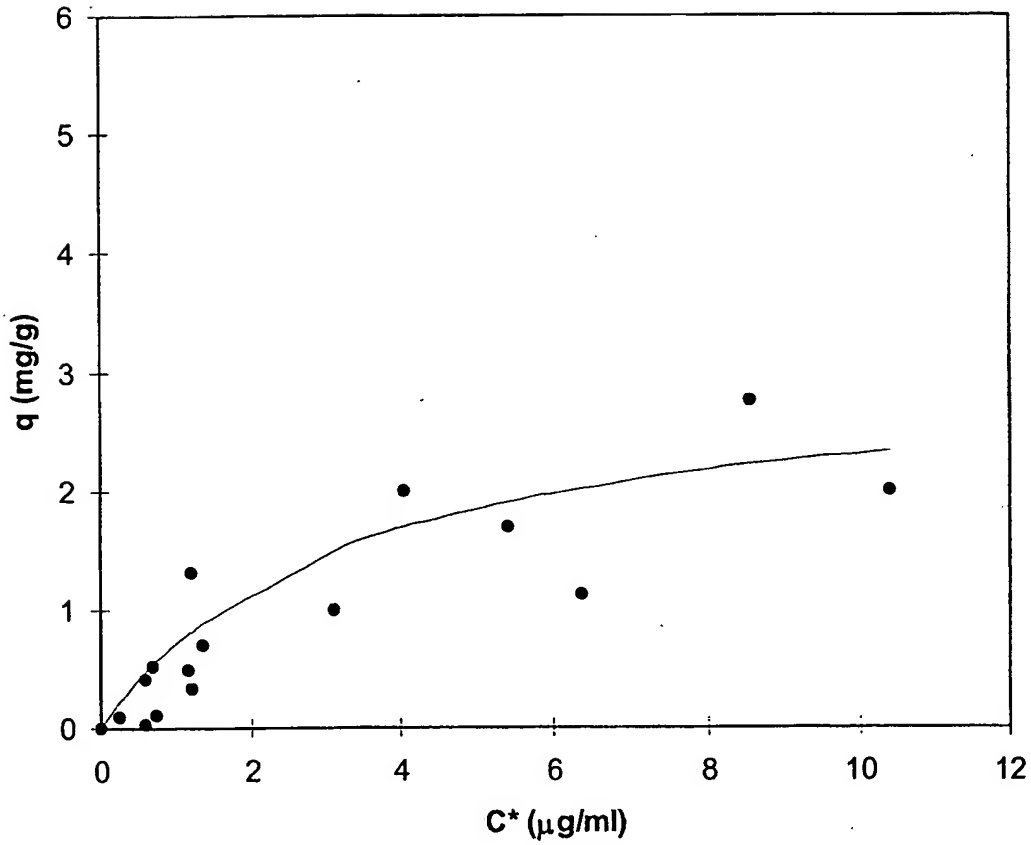


FIGURE 4

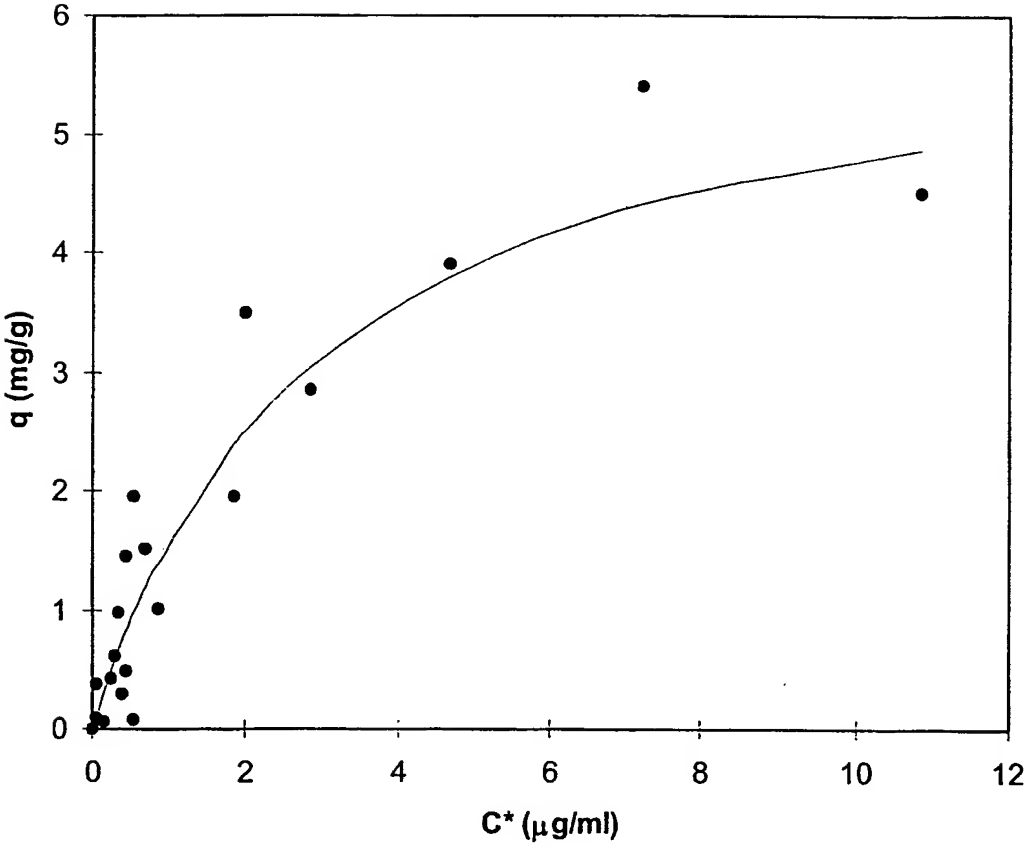
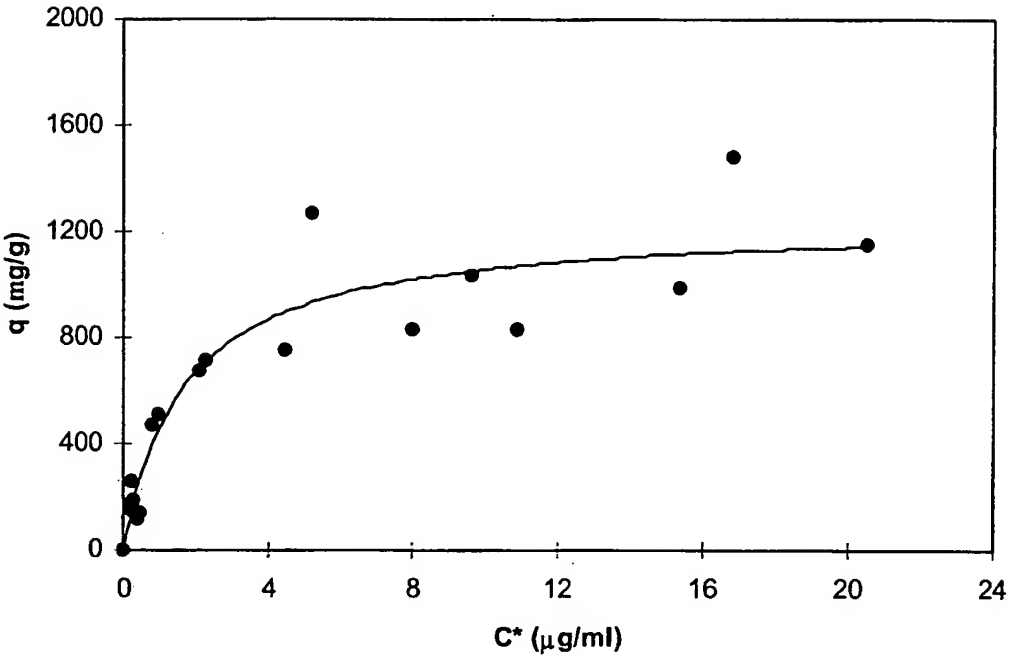


FIGURE 5



INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/PT 00/00009

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	US 4 699 717 A (RIESNER DETLEV ET AL) 13 October 1987 (1987-10-13) column 3 -column 4; figure 1; examples 1,2,6 ---	1-4,6-9
X	EP 0 268 946 A (DIAGEN GMBH) 1 June 1988 (1988-06-01) page 4 -page 5 page 7 --- -/--	1-3,6-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
8 December 2000	21/12/2000

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

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PCT/PT 00/00009

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	COLPAN M ET AL: "HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HIGH-MOLECULAR-WEIGHT NUCLEIC-ACIDS ON THE MACROPOROUS ION EXCHANGER NUCLEOGEN" JOURNAL OF CHROMATOGRAPHY, vol. 296, 1984, pages 339-354, XP000960881 ISSN: 0021-9673 page 346 -page 347	4,5
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P,X	FERREIRA GUILHERME N M ET AL: "Studies on the batch adsorption of plasmid DNA onto anion-exchange chromatographic supports." BIOTECHNOLOGY PROGRESS, vol. 16, no. 3, May 2000 (2000-05), pages 416-424, XP000972518 ISSN: 8756-7938 abstract; figure 1	1,2

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatio application No
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